Development of low Arsenic (As) accumulating Indica rice lines using genome editing approach

A

Synopsis

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INTRODUCTION

Arsenic (As) is found in various minerals forms, usually with sulfur and metals, and also found in two inorganic forms, arsenite [As (III)] and arsenate [As (V)]. Nearly, 105 countries worldwide are suffering from underground water As contamination (Chakraborti *et al.,* 2017). Anthropogenic and geogenic activities are sources of As contamination of drinking water due to which a large number of populations experience the ill effects of As toxicity. This issue is particularly genuine in developing nations like South America and Southeast Asia (Brammer and Ravenscroft, 2009; Mirlean *et al.,* 2014). In Bangladesh, agricultural fields were irrigated with As contaminated ground water which lead to accumulation of 1000 tons of As in the soil (Zhao *et al.,* 2010).

Since, 1988-groundwater As contamination has been reported in Indian states like Jharkhand, West Bengal, Uttar Pradesh and Bihar in Ganga flood plains, Manipur and Assam in Brahmaputra and Imphal rivers. The surface water arsenic contamination in five different districts including Ghaziabad, Moradabad, Mathura, Gorakhpur, and Agra of Uttar Pradesh has been reported (Tantry *et al.,* 2019). Analyses of 50 surface and tube well water samples have revealed the fact that district Agra showed highest arsenic concentrations, i.e., 0.242 mg/L in ground water. In surface water As level ranging from 0.556 mg/L to 0.342 mg/L, was observed excessively high as compared to the recommended limit (10 ppb or 0.01 mg/L) by World Health Organization (WHO) and Bureau of Indian standards (BIS).

As accumulation in soil is usually a result of herbicidal or insecticidal applications that contain As, mining activities, Arsenic containing underground water irrigation (Verbruggen *et al.,* 2009), using municipal solid waste as fertilizer. In humans, As (III) and As (V) are highly cytotoxic metalloid causing hyperpigmentation, skin lesions, diabetes, keratosis and other serious condition like cancer (Ghosh *et al.,* 2015). Contaminated underground water used for irrigation and drinking purpose (Smith *et al.,* 2002) is a major cause of As-related diseases.

Rice being a staple food is consumed by half of the world's population. Also, the rice grains assimilates As from the paddy soil; therefore, rice is primarily a good source of inorganic As (Li *et al.,* 2011).In comparison to other crops like barley and wheat, As accumulation in rice grain and shoots is

higher (Su *et al.,* 2010). The efficiency increases with increase in bioavailability of As as in flooded fields (Paddy fields) and the proficient As uptake system in rice.

The mineral nutrients such as silicon (Si), phosphorous (P), iron (Fe) and sulphur (S) can change the exchangeable fraction of As that can minimize the As concentration in soil. Si interaction with As has received attention in past years (Ma *et al.,* 2008; Saud *et al.,* 2016). In the anaerobic paddy field, As is mostly present as arsenite ($AsO3^{-3}$), which is taken to the roots by LSi1 transporters (nodulin 26-like intrinsic aquaporin) and effluxed towards the stele for xylem loading by silicon transporter Lsi2. Although other transporters are likewise ready to move arsenite (Ma *et al.,* 2008), Lsi1 and Lsi2 are the significant transporters of arsenite in rice because of their high expression in the roots (Ma *et al.,* 2008). Also, the cell localization of Lsi1 and Lsi2 empowers rice to take up Si and As more proficiently than other grain crops (Ma *et al.,* 2008; Mitani *et al.,* 2009). After arsenite uptake by the root cells, some of it is quickly discharged into the rhizosphere, a procedure that is somewhat intervened by Lsi1, a bidirectional channel (Zhao *et al.,* 2010a). The remaining As is sequestered into the root vacuoles or translocated to the shoots and further to different organs (Zhao *et al.,* 2010b). Phloem cells in the nodes contain elevated levels of As and seem to assume significant role in conveying As to the grains (Moore *et al.,* 2014), proposing that this tissue may sequester As and in this manner reduces delivery of As to the grain. Since rice grains are a significant source of As intake (Meharg *et al.,* 2009), reduction of As in the grain is a significant target of endeavors to decrease As toxicity. A physiological report indicated that transport of arsenite to the grain happens for the most part by means of phloem (Carey *et al.,* 2010). Modifying these transporter genes may reduce As content in rice. Thus, the elimination of As build-up in rice shoot and grain are the major research goal for the food safety and promoting human health.

In recent years, several gene editing technologies have been developed to genetically modify plants. Among them, CRISPR-Cas9 (Clustered regularly Interspaced Short Palindromic Repeats) system has been widely used in crop improvement and other applications because of its simplicity, versatility and efficiency. The technique is based on RNA mediated nuclease system present in bacteria and archaea which builds an adaptive immune system against viral infection which is based on the principle of Watson-Crick base pairing. Cas9 nucleases along with an engineered guide RNA

are the basic component of CRISPR-Cas system. As CRISPR-Cas system requires only single RNA to generate target specificity, it is more efficiently used for gene editing as compared to other synthetic nucleases developed.

The relevance and expected outcome of the proposed study

Groundwater contamination with Arsenic [As (III)] is a major concern in India and other countries. Underground water is a primarily source of irrigation by which As is accumulated in various crops including rice, causing severe health issues in humans. Treatments like water management practices and increasing other minerals in the soil can alter the As uptake and availability to the plants (Farooq *et al.,* 2016). However, these methods are not long term solutions because As can be remobilized with change in soil conditions. Therefore, selection and breeding of low As accumulation rice variety is an economical strategy that may allow rice to grow in As-polluted fields.

- The present study will be focused on developing a way to minimize the uptake of Arsenic in rice using genome editing.

Objectives:

Arsenic accumulation in rice is a major problem in different parts of the world and development of rice with reduced Arsenic uptake is needed. In view of this the present work has been taken in the following steps:

1. Identification of LSi1 gene for high As uptake in rice

1.1 Screening of Rice varieties for differences in As accumulation

1.2 Generation of random Lsi1 mutant

2. Generation of low-As accumulating rice using CRISPR Cas9 system.

2.1 Preparations of vector construct.

2.2 Development of rice transgenics.

3. Determination of metal concentration of the mutant lines.

REVIEW OF LITERATURE

The development of elite rice cultivars and other crops has been a challenge for conventional approaches, and new strategies urgently need to be developed. Since 2013, after knowing the efficiency of CRISPR technique in *Arabidopsis* (model plant), and *Nicotiana benthamiana* (Tobacco),

it has been used in other crops like rice (Zhnag *et al.,* 2014), sorghum (Jiang *et al.,* 2013), and wheat (Wang *et al.*,2014).

Rice grains accumulate Cadmium (Cd) through soil which is a toxic metal. Developing a rice cultivar with low Cd content was a challenge to the conventional breeders so with the emerging technology of CRISPR Tang *et al.,* (2017) knocked out OsNramp5 resulting in production of low Cd-accumulating rice cultivars without compromising the yield. Hydroponic culture of osnramp5 mutants showed decrease in Cd concentration to an extent which resulted in growth of rice plants in high Cd concentrated soil and also developed a rice line with low Cd concentration in grains. Paddy fields with Cd contamination was checked and found that osnramp5 mutants have less than 0.05 mg/Kg of Cd to 0.33 mg/kg to 2.90 mg/kg in wild type indica rice.

Cesium (Cs) in food has become a concern after nuclear incidents. Even at very low concentration (below µM) can be taken up by plants and accumulate in their edible parts. Several strategies to reduce Cs+ from soil are in practice but with no longer impacts. A biotechnological approach was opted for reduction of Cs^+ accumulation in rice plant using CRISPR/Cas. A Cs^+ permeable K+ transporter OsHAK1was targeted and resulted in reduction of Cs^+ in Rice plants. OsHAK1 expressed in both rice and *Saccharomyces* (Yeast cells) showed similar kinetic results (NievesCordones *et al.,* 2017).

In a study thermo-sensitive genic male sterility (TSMS) gene has been targeted using CRISPR/Cas system in rice and developed transgene free TGMS lines. Ten Target sites were selected in the coding region of TMS5 for mutagenesis. TMS5ab construct was selected for breeding transgene clean rice lines. This showed the application of the CRISPR system in accelerating breeding sterile lines (Zhou *et al.,* 2016).

Sun *et al.,* (2017) generated high-amylose rice by targeting the starch binding enzyme (SBE). SBEI and SBEIIb were targeted in which SBEIIb significantly increased amylose content (AC) and resistant starch (RS) in rice. The frequency of homozygous mutants found was from 26.7% to 40% and the homozygousmutants in T_0 generation were stably transmitted to T_1. Result showed the feasibility of the system to develop high amylose content in rice.

Plant annexins are phospholipid binding proteins (Ca-dependent) useful for plant development and protection from environmental stresses. Knockout of this annexin gene OsAnn3 using CRISPR/Cas9 decreases cold tolerance in rice (Shen *et al.,* 2017) suggesting the involvement of OsAnn3 in cold tolerance. Mutant plants obtained were tested for cold tolerance and found that T_1 mutant's survival ratio was low due to knockout of the gene.

Rice bran oil obtained from rice which contains oleic acid has several health benefits. Fatty acid desaturase 2 (FAD2) is an enzyme for the conversion of oleic acid to linoleic acid in plants. Three functional genes of FAD2 are present amongst which OsFAD-1 gene shows high expression in rice seeds. So, the idea was to disrupt the OsFAD-1 gene to obtain high oleic/low linoleic content. Recently, Abe *et al.,* (2018) succeeded in producing a rice variety with improved fatty acid composition. OsFAD2-1 gene (fatty acid desaturase) was disrupted by CRISPR-Cas9 system which resulted in increased linoleic content to more than twice of wild type and decrease in linoleic acid to undetectable levels.

Three homologous genes of FAD2 have been targeted using CRISPR/Cas9 in *Camelina* seeds which resulted in enhanced fatty acid composition. FAD2 gene in both (*Arabidopsis* and *Camelina*) closely related plants were targeted with an incentive to increase oil composition. In allohexaploid (*Camelina*) sgRNA was designed to target the three homologous FAD2 genes which resulted in increased oil composition (T_3 and T_4). Oleic acid content was increased from 16% to 50% as reported by Jiang *et al.,* (2017).

Yu *et al.,* (2017) generated a tomato line with long shelf life using CRISPR/Cas9 based mutagenesis. ALC gene was mutated and replaced using *Agrobacterium* mediated CRISPR/Cas system of transformation. The mutation frequency obtained was 72.3% in T_O generation. The mutants obtained were further confirmed by genotypic and phenotypic characterization which showed good shelf life.

Rice production is affected by abiotic stress all around the world. Zhang *et al.,* 2019 reported an improvement in rice salinity tolerance using engineered expression vector (Cas9-OsRR22-sgRNA) targeting the OsRR22 gene. Among 14 transgenics nine mutants were obtained having six mutation

types successfully transferred to the next generation. Results concluded that CRISPR/Cas9 can be a useful method to increase salinity stress tolerance in rice.

A new rice mutant has been developed with cold tolerance and high yield by editing multiple genes, GS3, OsPIN5b and OsMYB30 with CRISPR technology. The mutants of OsPIN5b, OsMYB30 and GS3 resulted in increased panicle length, increased cold tolerance and enlarged grain size, respectively (Zeng *et al.*, 2020).

Biotic stress is also a major problem in rice crop. Wang *et al.*, (2016) targeted the OsERF922 gene in rice to improve rice blast resistance. Twenty one ERF922 mutants were identified among 50 transgenic developed in T_0. It was reported that all the C-ERF922 allelic mutations were transmitted to next generation. Also, multiple sites were targeted (OsERF922) using Cas9/Multi-target-sgRNA resulting in mutations at two or three sites.

Kaur *et al.,* 2017 demonstrated a successful mutation in phytotene desaturase (PDS) and precise manipulation in banana genome. Two genes of RAS-PDS has been targeted and transformed with Cas9 in embryonic cultures of cv. Rasthali. Chlorophyll and total carotenoid contents were decreased in the mutant lines showed the functional disruption of both the genes. In India, CRISPR/Cas related work is still in infancy stage.

By reviewing the literature, it is concluded that gene modification through CRISPR/Cas is useful approach to enhance crop productivity, resistance to biotic and abiotic stress and nutritional value. So as it can be helpful in creating a rice line with low accumulation of Arsenic.

PROPOSED METHODOLOGY

1. **Identification of LSi1 gene for high As uptake in rice**

1.1 Screening of Rice varieties for differences in As accumulation:

   a. Collection of rice seeds from different regions will be grown on MS medium.
   b. Arsenic treatment to identify high As accumulating rice will be selected for further study.
   c. Screening and sequencing will be helpful in identifying gene that uptake less As as a reference for genome editing.

1.2 Generation of random Lsi1 mutant: Site-directed random mutagenesis in Lsi1 using a method

(Fromant *et al.*, 1995) will be performed.

2 Generation of low-As accumulating rice using CRISPR Cas9 approach.

Based on the gene obtained from Objective 1 responsible for low As uptake will be used to design sgRNA.

2.1 Preparations of vector construct

a. *Selecting Cas9 target site:* Based on CRISPR/Cas9 design system 19-20 bases upstream of PAM sequence will be selected as target sequence. To confirm the target specificity in the rice genome, target sequence (including PAM) will be BLAST against the whole rice genome and also CRISPR-P program can be used to minimize the off-target effects of selected sequence.

b. *Construction of sgRNA expression vector and rice transformation:* The sgRNA expression vector will be constructed in such a way that it can swap with the target sequence easily.

2.2 Development of rice transgenics

a. *Validation of sgRNA by transient expressions:* Validation of sgRNA activity is necessary and will be done by co-transforming pOsU3-sgRNA with pJIT163-2NLSCas9 into rice protoplast for transient expression.

b. Transformation will be done by introducing binary construct into *Agrobacterium tumefaciens* (Ranjan *et al*., 2012).

c. *Mutation detection and evaluation of T_o plants:* DNA will be extracted from the leaves during its maturity period by a modified CTAB method (Zhang *et al.,* 2014). CRISPR/Cas9 mutants will be screened using a sensitive method based on PCR (Hua *et al.*, 2017)

3. Determination of metal concentration of the mutant lines.

Evaluation metal ion (As) concentration: To determine the As concentration in the soil, samples will be digested using microwave digestion method. Arsenic concentration will be determined and analysed using an inductively coupled plasma mass spectrophotometer (ICP-MS) (Roychowdhury *et al.*, 2005)

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